AD_____

AWARD NUMBER: W81XWH-15-1-0161

TITLE: Gene-Specific Demethylation as Targeted Therapy in MDS

PRINCIPAL INVESTIGATOR: Daniel G. Tenen, M.D.

RECIPIENT: Beth Israel Deaconess Medical Center, Boston
Boston, MA 02215-5491

REPORT DATE: July 2016

TYPE OF REPORT: annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
July 2016	Annual Report	15 Jun 2015 - 14 Jun 2016

4. TITLE AND SUBTITLE
Gene-Specific Demethylation as Targeted Therapy in MDS

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0161

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Daniel G. Tenen, MD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
330 Brookline Avenue
Boston, MA
02215-5491

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Material Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*

Beth Israel Deaconess Medical Center

Harvard Medical School
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July 29, 2016

Department of Defense (DoD) Office of Congressionally Directed Medical Research Programs (CDMRP)
Bone Marrow Failure Research Program (BMFRP) Idea Development Award

Re: W81XWH-15-1-0161: "Gene Specific Demethylation as Targeted Therapy In MDS"

Dear Sir/Madam:

Attached is a revised Progress Report as requested by our Science Officer, Dr. Shui-Lin Niu. As it took some time to obtain proper Human Studies approval, we are initiating the experiments involving human studies in Year 2 of the award. The revised Progress Report describes experiments that did not involve human studies. As discussed with Dr. Niu, we have attached a revised Statement of Work (SOW) at the end of this report.

Sincerely,

Daniel G. Tenen
Principal Investigator

Julienne Carty
Research Administrative Director
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330 Brookline Ave. E/CLS650
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Tel: 617-735-2002
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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Myelodysplastic Syndromes (MDS) are a group of clonal hematopoietic disorders characterized by bone marrow failure and risk of progression to Acute Myeloid Leukemia (AML) in approximately 30 percent of the cases. Aberrant DNA methylation is considered a dominant mechanism for Tumor Suppressor Genes silencing during MDS evolution to AML, but the causes leading to aberrant DNA methylation remain elusive. This proposal builds on our recent discovery of a novel class of RNAs, the DiRs or **D**NMT1-**i**nteracting **R**NAs, involved in cell type-specific DNA methylation patterns. We have found that DNMT1 binds to RNA with stronger affinity than DNA of the same primary structure. This interaction inhibits DNMT1 enzymatic activity thereby preventing DNA methylation and the resultant silencing of the corresponding DiR-regulated gene loci. Based on these findings, we hypothesize that DNA methylation changes can be corrected by RNAs. We aim to demonstrate that: a) by inducing transcription within targeted methylated genomic loci or b) by utilizing oligonucleotides mimicking the function of DiRs and able to specifically target methylated loci, we will be able to reduce level of methylation and consequently rescue the expression of the respective silent gene. In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Myelodysplastic syndrome; p15, DNA methylation; RNA

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

In this proposal we plan to apply these approaches to yet another gene, *P15* (*CDKN2B*), an important gene exploiting not only cell-cycle regulator functions, but revealing specific features in the regulation of hematopoietic progenitor cell fate. *P15* is the gene most frequently silenced by aberrant promoter methylation in MDS and it is associated with poor prognosis and increased risk of transformation to AML. Therefore, we propose the following two aims: **Aim 1.** *To reduce P15 locus specific genomic methylation by induction of its respective DiR;* **Aim 2.** *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1. *To reduce P15 locus specific genomic methylation by induction of its respective DiR*

Our previous studies demonstrated that downregulation of *ecCEBPA* led to decreased *CEBPA* mRNA and increased DNA methylation levels, whereas ectopic expression of *ecCEBPA* resulted in an opposite outcome. Further, we proved that RNA has a stronger affinity than DNA to DNMT1 and that RNA specifically interacts with the DNMT1 catalytic domain leading to the hypothesis that RNA oligonucleotides could be utilized as gene specific demethylating agents. We continued exploring this avenue and chose as a model for this study two tumor suppressor genes chromosomally linked and frequently methylated in cancer: the *CDKN2A* (aka *P16*) and *CDKN2B* (aka: *P15*). While *P16* is commonly methylated in solid tumors, *P15* is silenced in myeloid disorders. Therefore, we decided to apply the same RNA-mediated demethylating approach to both genes. During, the first funded year, we have established and optimized the conditions to perform RNA-FISH using the *CEBPA* gene locus, and now we will proceed with the mapping and localization of *P15/ P16* and the respective DiR-like transcripts on cell lines expressing both genes.

We have demonstrated that *ecCEBPA* transcription is initiated in the early S phase and preceeds the expression of *CEBPA* mRNA. In an attempt to identify all the potential transcripts with features similar to *ecCEBPA* (enriched in the nucleus and transcribed during the S-phase), including those corresponding to the *P15* and *P16* loci, we performed nuclei RNA isolation, RNA purification, and deep sequencing and have combined this approach with the Click-iT® technology. This method is based on the bioorthogonal click chemistry reaction, which enables the metabolic incorporation of ethynyl uridine (EU), a "clickable" ribonucleoside, into RNA during nascent RNA synthesis. Briefly, biotin is “clicked” onto the nascent chain and strepavidin magnetic beads capture all newly synthesized transcripts. To compare the transcriptional profiles under these conditions with our previous results, we performed RNA-seq on total and nuclear RNA fractions of unsynchronized and S-phase synchronized HL-60 cells. Although HL-60 lacks both P16/P15 proteins, the entire locus displays antisense transcription of the long non-coding RNA antisense to *P15*: ANRIL, reported as a *P15* silencer. ANRIL seemed expressed at the highest levels during the S-phase in the nuclear compartment, suggesting a potential counteracting effect on the DiR-like transcripts arising from the locus. We have now designed a mapping strategy to characterize both expressing and not-expressing cell lines. Overall, these results suggest a complex regulation of the locus that can be dictated by a fine-tuning of sense and antisense transcription and enhance the relevance of RNA as a therapeutic tool to control gene expression.

Aim 2. *To reduce P15 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the P15-DiR.*

The goal of this aim is to evaluate whether RNA can be repurposed as a gene-specific demethylating tool to correct aberrant DNA methylation. We applied two distinct approaches to fulfill this aim. The first approach entails the use of a triplex forming RNA oligonucleotides (TFO) of approximately 25 bases fused to a 25 bp RNA sequence from *ecCEBPA*, which was shown to bind to *ecCEBPA* by REMSA. The TFOs were selected based on the prediction that they

would form a triplex structure with the locus being targeted. The second approach utilized a CRISPR/dCAS9 system where the guide RNA includes the *ecCEBPA* DNMT1 "bait" sequence. Briefly, we fused the RNA stem loops (R2 and/or R5), interacting with DNMT1(1) to the tetra and/or stem loop 2 in sgRNA scaffold to obtain a modified sgRNAs (MsgRNA). We started our approach targeting the *P16* promoter in a hepatocellular carcinoma (HCC) cell line SNU-398, easy to transfect, and in which *P16* is silenced by promoter methylation. 72 hours after transfection, we observed a 3-fold increase of *P16* mRNA by qRT-PCR in the cell line treated with the MsgRNA. We also observed loss of DNA methylation of the locus compared to cells transfected with unmodified gRNA, confirmed by bisulfite sequencing. No changes of mRNA levels for the adjacent *P15* gene were observed, supporting a gene-specific demethylating activity of the approach. In order to trace the dynamic relationship between DNA methylation, re-methylation, chromatin changes, and gene expression, we established a cell line stably expressing dCas9 with a tetracycline/doxycycline-inducible MsgRNA. In parallel, we adopted an *in-vitro* cleavage assay (NEB) to further improve the MsgRNAs design and optimize the target of the chosen loci. Currently, we are developing a methylation reporter assay to study the stability of the DNMT1-RNA complex formation, based on the structure of the MsgRNA. This type of screening should allow us to select the most effective and strongest guides to utilize with the inducible Crispr/dCas9 system and to potentiate the gene-specific demethylation action. Similar approaches will be also pursued for the *P15* locus and are currently ongoing. In particular, at the moment we are exploring an additional avenue of activation entailing the usage of small RNA oligonucleotides, represented by the short-activating RNAs, specifically designed for the *P15* locus.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next reporting period, we will continue the experiments as planned in the two aims. We will clone optimized DiRs designed to induce demethylation of P15 or P16 into lentiviral vectors, and develop cell lines which can inducibly express these DiRs to determine whether we can activate either P15 or P16, respectively. We will also measure global effects in these lines, measuring changes in DNA methylation and gene expression to determine if genes other than the targets (P15 or P16) are affected.

Secondly, we will design and synthesize triplex forming oligonucleotides (TFOs): composite DNA oligonucleotides (CDOs); and chimeric RNA oligonucleotides (CROs) and test their ability to form triplexes with their target DNAs, namely P15 and P16. We will test and optimize methods for delivery of these into cell lines, and begin to analyze the lines we produce.

With the approval of the human studies protocol, we will now initiate studies to optimize delivery of TFOs into primary cells..

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Patients with MDS respond to demethylating drugs, but the effects are limited, possibly due to the effects of general demethylation. We have initiated studies to develop methods to use RNA to induce gene specific demethylation of genes that are downregulated in MDS, in order to restore normal blood production.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The techniques being developed can be used to demethylate and activate silenced genes in other cancers or other diseases.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

A major delay was obtaining human studies approval. We first had to obtain an amendment of our approved protocol from the Beth Israel Deaconess Medical Center IRB, which has now been obtained. We have now obtained approval for the human studies from the Department of Defense.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Because we were told not to initiate studies until obtaining human studies approval, initiation of the project was delayed and therefore expenditures less than anticipated. However, we were able to initiate a number of experiments that did not involve and human studies.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No changes. Human studies have now been approved.

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

  Presentations:

  "Regulation of DNA methylation by noncoding RNA", Boston University School of Medicine, Whitaker Cardiovascular Institute, January 26, 2016.

  "Epigenetic regulation by RNA in a normal and malignant context", University of Birmingham, UK, February 19, 2016.

  "Regulation of myeloid transcription factors, DNA methylation, and leukemia by noncoding RNA", National Institutes of Health, Bethesda, March 22, 2016.

  "Noncoding RNA regulation of myeloid transcription factors, DNA methylation, and leukemia", Weatherall Institute of Molecular Medicine, Oxford University, UK, April 29, 2016.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  None

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*

- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Daniel G. Tenen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6423-3888
Nearest person month worked:	2.4
Contribution to Project:	Dr. Tenen is overseeing the direction of the project
Funding Support:	Other grants (see below)
Name	Junyan Zhang
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms Zhang assists Dr. Tenen in this project
Funding Support:	This grant and other NIH grants (see below)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Since the submission of this proposal in August, 2014, one active grant has ended:
R01 HL112719-28, entitled "Molecular biology of myeloid differentiation", which focused on the study of the regulation of the PU.1 transcription factor.

In addition, three proposals were funded:

1R01DK103858-01 (Tenen, PI). "Noncoding RNA-DNMT1 interactions in hematopoiesis". This grant is in response to PAS13-031, Stimulating Hematology Investigation: New Endeavors (SHINE) (R01). The major goals of this proposal are to understand the role of ecCEBPA in hematopoietic differentiation.

P01 CA66996-16 (Ebert, PI). Development of Novel Therapeutic Strategies in Human Myeloid Leukemia. Project 3: Lysine acetyltransferases in myelopoiesis and leukemia. The overall goal of this project are to determine the role of acetylation in the function of the myeloid tumor suppressor C/EBPa. The specific aims are: Aim 1: To investigate the role of acetylation of C/EBPa in granulopoiesis; Aim 2: To target post-transcriptional modifications of C/EBPa in leukemia.

1R35CA197697-01 (Tenen, PI). "Mechanisms of regulation by RNA in acute myeloid leukemia"
The major goals are to investigate mechanisms through which RNAs contribute to gene dysregulation in acute myeloid leukemia, including the role of long antisense RNAs and RNA editing in core binding factor leukemias.

These changes do not impact the effort on the project being reported on.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None

STATEMENT OF WORK
START DATE September 30, 2015

Site 1: Beth Israel Deaconess
330 Brookline Ave, Boston 02215
PI: Daniel G. Tenen

Specific Aim 1(specified in proposal) To reduce P15 locus specific genomic methylation by induction of its respective DiR.	**Timeline**	**Site 1**
Major Task 1 : Design and cloning of the vectors expressing the p15/p16 gene locus DNMT1-interacting RNAs.	Months	
Subtask 1: 5'3' RACE for PCR amplification of the p15/16 flanking regions.	6-9	Dr. Zhang
Subtask 2: DiRs overexpression of both 5' and 3' ends of the P15/P16 gene in the EGFP-lentiviral vector pWCC19.	9-12	Dr. Zhang
Major Task 2: Producing lentiviral particles and transduction		
Subtask 1: Producing lentiviral particles and transduction into cell lines	9-12	Dr. Zhang
Subtask 2: Analysis of the produced cell lines, including effects of DiR overexpression on the P15/P16 gene and global effects, measuring methylation and gene (mRNA) expression.	12-24	Dr. Zhang
Subtask 3: Overexpression of the P15/P16-DiRs in primary cells, and their analysis.	25-30	Dr. Zhang
Subtask 4: Analysis of overexpression of the P15/P16-DiRs in primary cells	30-36	Dr. Zhang
Milestone(s) Achieved: Demonstration that overexpression of a p15/p16-DiR can induce demethylation of the p15/p16 locus and activate P15/P16 mRNA expression, in both cell lines and primary cells.	1-36	Dr. Zhang
Specific Aim 2: To reduce P15/P16 locus specific DNA methylation by introduction of oligonucleotides mimicking the action of the DiRs		

Major Task 3: Design and synthesis of the triplex-forming oligonucleotides (TFOs): composite DNA oligonucleotides (CDOs) and chimeric RNA oligonucleotides (CROs)		
Subtask 1: Design and synthesize triplex-forming oligonucleotides (TFOs)	9-12	Dr. Zhang
Subtask 2: , Test CDOs and CROs in vitro by the ability to form triplexes with target genomic sequences by EMSAs/REMSAs	12-18	Dr. Zhang
Milestone(s) Achieved: Identify the best candidates for subsequent cellular studies	1-18	Dr. Zhang
Major Task 4: Delivery and analysis of the TFOs into cell lines		Dr. Zhang
Subtask 1: Identify optimal methods for delivery	12-18	Dr. Zhang
Subtask 2: Analysis of the transfected cells lines, including methylation and mRNA expression of p15/p16, as well as global effects on other genes	18-36	Dr. Zhang
Milestone(s) Achieved: Optimize delivery of TFOs into cells lines such that p15/p16 is selectively demethylated accompanied by induction of p15/p16 mRNA	12-36	Dr. Zhang
Major Task 5: Delivery and analysis of the TFOs into primary cells		Dr. Zhang
Subtask 1: Identify optimal methods for delivery	18-30	Dr. Zhang
Subtask 2: Analysis of the transfected cells, including methylation and mRNA expression of p15/p16, as well as global effects on other genes	31-36	Dr. Zhang
Milestone(s) Achieved: Optimize delivery of TFOs into primary cells such that p15/p16 is selectively demethylated accompanied by induction of p15/p16 mRNA	18-36	Dr. Zhang

If human subjects are involved in the proposed study, please provide the projected quarterly enrollment in the following table.

	Year 1				Year 2				Year 3			
Target Enrollment (per quarter)	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Site 1	0	0	0	0	3	3	3	3	6	6	6	6
Target Enrollment (cumulative)	**0**	**0**	**0**	**0**	**0**	**6**	**9**	**12**	**18**	**24**	**30**	**36**